# The dynamic response of upstream DNA to transcription-generated torsional stress

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**The torsional stress caused by counter-rotation of the transcription machinery and template generates supercoils in a closed topological domain, but has been presumed to be too short-lived to be significant in an open domain. This report shows that transcribing RNA polymerases dynamically sustain sufficient torsion to perturb DNA structure even on linear templates. Assays to capture and measure transcriptionally generated torque and to trap short-lived perturbations in DNA structure and conformation showed that the transient forces upstream of active promoters are large enough to drive the supercoil-sensitive far upstream element (FUSE) of the human *c-myc* into single-stranded DNA. An alternative non-B conformation of FUSE found in stably supercoiled DNA is not accessible dynamically. These results demonstrate that dynamic disturbance of DNA structure provides a real-time measure of ongoing genetic activity.**

Counter-rotation of the transcription machinery and template as DNA is threaded through the RNA polymerase active site positively supercoils DNA in front of the enzyme as negative supercoils trail[1,2]. Within fixed topological boundaries, these torsional forces would rapidly rise to deleterious levels unless dissipated[3,4]. However, lower levels of supercoiling may contribute to regulatory phenomena. Negative supercoiling destabilizes DNA in a context- and sequence-dependent manner, potentially favoring non-B conformations. By altering its mechanical and elastic properties, supercoiling can facilitate or impede DNA transactions including juxtaposition of factors bound at remote sites or the tracking of molecular machines (such as polymerases or helicases) along the double helix[5,6]. Torsional forces can also influence chromatin stability as remodeling machines may be assisted or hindered by supercoiling[7]. Topoisomerases are the main agents draining torsional stress from the genome. At the level of single genes, the microarchitecture of chromatin loops, the interactions among DNA-bound proteins, and the placement of topoisomerases would all influence the extent of local supercoiling[3,4,8].

Lacking topoisomerase or nuclease action, the linking number, a measure of the intertwining of DNA strands relating helical structure, supercoiling and DNA melting, is constant within a fixed domain[3,4,8]. Chromatin is dynamic, however, and as molecular motors push, pull and twist DNA, transient torques develop even in topologically open systems[9,10]. Do these evanescent stresses decay too rapidly to influence genetic processes (unless captured and stored in fixed domains) or can molecular motors such as RNA polymerase pump torsional energy into DNA and chromatin rapidly enough to sustain or assist conformational changes even in topologically open systems? Such dynamical perturbation of DNA or chromatin could enable real-time sensing of ongoing genetic activity or mechanically link the activity of adjacent genes. Although this phenomenon has long been anticipated or observed in experiments using topologically bounded templates[9–13], until now dynamic supercoiling has not been directly measured.

We sought a system to measure the torsional stress that is dynamically generated during the transcription of a linear template *in vitro*. The human *c-myc* gene FUSE is an example of a DNA sequence that hypothetically couples DNA topology with transcription. The FUSE sequence (90 base pairs (bp)) has been predicted to melt with high probability in response to supercoiling[14] and contains a 22-bp quasi-mirror repeat sequence that might form H-DNA (a folded-back triple-helical kink with a single-stranded segment[4]). Single-stranded FUSE engages the FUSE-binding protein (FBP) to modulate *c-myc* expression[14–17]. If FUSE conformation were sensitive to transcriptional torque, it would report nearby promoter activity in real time. The transcriptional effector domains of FBP might then provide immediate feedback on *c-myc* transcription. In this report, we show that FUSE undergoes a conformational change driven by the dynamic torsional stress of ongoing transcription. The magnitude of these transient forces was calibrated against the structural changes in FUSE when embedded in a series of progressively supercoiled circles. Moreover, the dynamic torsion of transcription transiting through topologically open DNA was directly trapped and found to rise to notably high levels. These results suggest that cells have all the components to assemble a mechanical feedback mechanism coupling the activity of transcription factors with ongoing transcription.
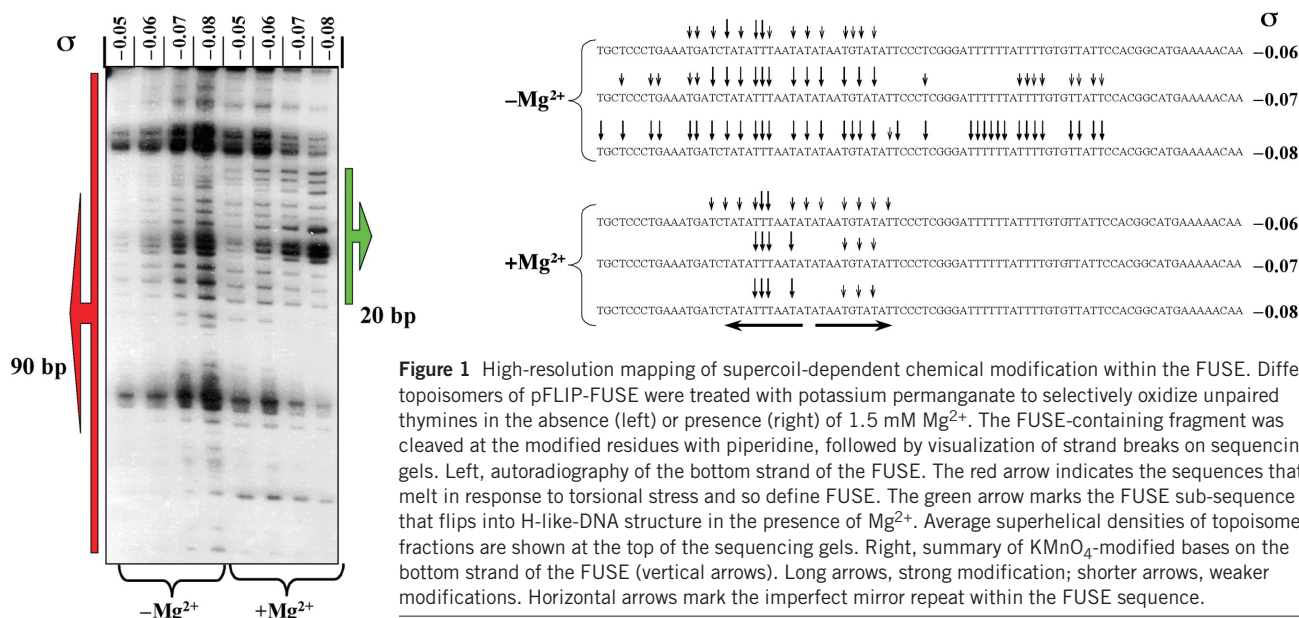
## RESULTS

### FUSE distortion measures torsional stress

Supercoil-driven structural transitions depend on the magnitude of the torque in the DNA. To use FUSE as a superhelical stress sensor, the

**Figure 1** High-resolution mapping of supercoil-dependent chemical modification within the FUSE. Different topoisomers of pFLIP-FUSE were treated with potassium permanganate to selectively oxidize unpaired thymines in the absence (left) or presence (right) of 1.5 mM $Mg^{2+}$. The FUSE-containing fragment was cleaved at the modified residues with piperidine, followed by visualization of strand breaks on sequencing gels. Left, autoradiography of the bottom strand of the FUSE. The red arrow indicates the sequences that melt in response to torsional stress and so define FUSE. The green arrow marks the FUSE sub-sequence that flips into H-like-DNA structure in the presence of $Mg^{2+}$. Average superhelical densities of topoisomer fractions are shown at the top of the sequencing gels. Right, summary of $KMnO_4$-modified bases on the bottom strand of the FUSE (vertical arrows). Long arrows, strong modification; shorter arrows, weaker modifications. Horizontal arrows mark the imperfect mirror repeat within the FUSE sequence.

theoretically predicted melting profile of this sequence was tested using potassium permanganate to modify unpaired or distorted bases (especially thymidine) as the average superhelical density, $\langle\sigma\rangle$, of the FUSE-bearing pFLIP-FUSE was increased from 0 to –0.12 (ref. 18). (Throughout this work the measurement of $\langle\sigma\rangle$ is ±0.005.) The FUSE region was excised from the treated molecules using restriction endonucleases, asymmetrically end-labeled, piperidine-cleaved at modified nucleotides and visualized by electrophoresis and autoradiography. Increasing $\langle\sigma\rangle$ in the absence of $Mg^{2+}$- induced FUSE melting within a mirror repeat, which spread to include all of the nearby thymidines (**Fig. 1**), exactly as predicted.

In the presence of $Mg^{2+}$, as $\langle\sigma\rangle$ increased, the diffuse and weak permanganate reactivity progressively intensified, focusing into the mirror repeat. The sharp transition in the cleavage pattern between $\langle\sigma\rangle$ = –0.06 and –0.08 was evocative of H-DNA formation[19]. The repeat's preponderance of unusually stable TAT-base triads would enhance divalent cation–dependent triplex formation even at neutral pH[3,20] where H-DNA is stabilized by divalent cations[3,4]. Thus, sustained torsional stress *in vivo* might drive FUSE into this non-B, $Mg^{2+}$-dependent conformation. $Mg^{2+}$ has been implicated in the supercoil-dependent stabilization of other non-B-DNA structures[21,22].

The transition from B- to non-B-DNA in a topologically bounded segment removes supercoils. Thus, a structural change in a small region of a closed circle necessitates a change in overall DNA topology. Two-dimensional gel electrophoresis was used to assess overall topology and relate changes in FUSE structure with particular thresholds of $\langle\sigma\rangle$[2,3]. In the first dimension, the topoisomers with a non-B-DNA segment overlap other topoisomers with lower $\langle\sigma\rangle$ that retain this segment as B-DNA. These distinct but comigrating topoisomers are resolved in the second dimension using an intercalator to absorb supercoils, thus reverting the non-B-DNA to normal duplex. A discontinuity in the two-dimensional topoisomer pattern indicates the number of helical turns locally unwound within the non-B-segment, and defines the superhelical stress driving the transition to the alternate structure.

To avoid competition with structural transitions at other sites (detected using $KMnO_4$ and S1 nuclease; data not shown), site-specific

Cre recombinase was used to excise FUSE-bearing DNA circles devoid of other sequences easily melted in response to supercoiling. +FUSE and –FUSE circles were compared using two-dimensional electrophoresis in the presence or absence of $Mg^{2+}$ (**Fig. 2**). Without $Mg^{2+}$ (**Fig. 2a**), the +FUSE circles beyond topoisomer –7 ($\langle\sigma\rangle$ = –0.07) that comigrated in the first dimension formed a broad plateau of four or five spots in the second. This plateau represents a family of molecules in which the number of supercoils is identical in the first dimension, but different in the second, and is best explained by the progressive unwinding of an easily melted segment, such as FUSE absorbing torsional stress and buffering changes in supercoils[24,25]. Each successive topoisomer within this plateau unwinds one more turn (10.5 bp) than the preceding topoisomer. At position A, the +FUSE DNA would unwind ~40 bp. At position B on the gel, a second plateau revealed a second melting segment further buffering changes in topoisomer mobility in the first dimension as $\langle\sigma\rangle$ was increased. No transitions were evident in the –FUSE 'midi-circle.' These results (**Figs. 1** and **2**) confirm the predicted, supercoil-driven biphasic melting of FUSE.

In the presence of $Mg^{2+}$, +FUSE and –FUSE circles traced the same curve through topoisomer –8 (**Fig. 2b**). A furrow in the +FUSE curve beginning with topoisomer –8 indicated a structural transition. Although they comigrated in the first dimension, topoisomer A and topoisomer –7 resolved by two supercoils in the second dimension. Thus a supercoil-dependent structural transition removed two supercoils in the first dimension from molecules migrating at position A. Flipping of FUSE's 22-bp quasi-mirror repeat into an H-like conformation would account for this relaxation (**Fig. 1**).

In summary, chemical probing and two-dimensional gel electrophoresis demonstrated that in the absence of $Mg^{2+}$, beyond $\langle\sigma\rangle$ = –0.07, the entire FUSE has the characteristics of melted DNA, but if stabilized by $Mg^{2+}$, the quasi-mirror repeat within FUSE adopts an H-like structure beyond the critical $\langle\sigma\rangle$.
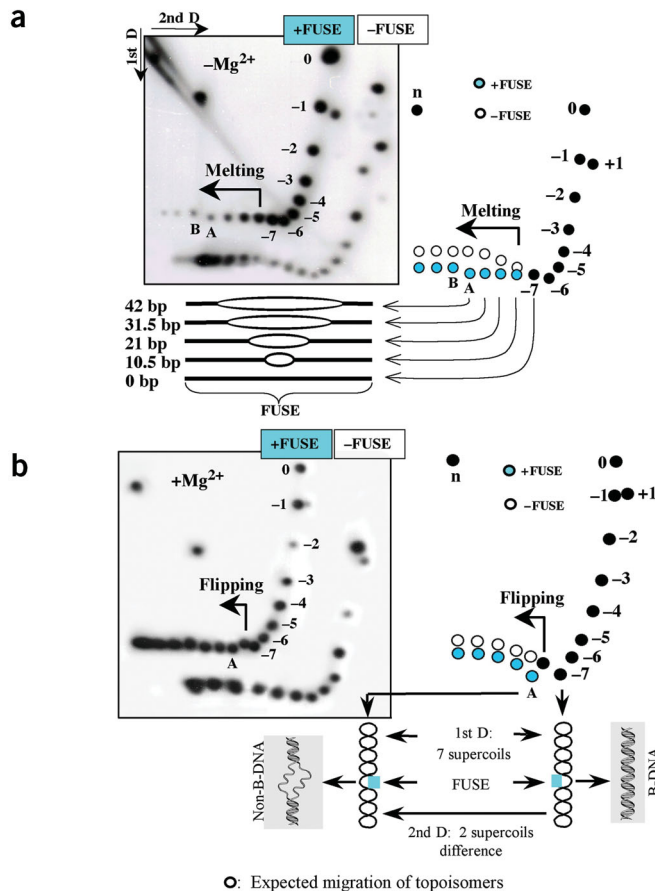
## Melting of FUSE by dynamic supercoils

To test whether ongoing transcription transmits enough torque to perturb DNA structure, even in a topologically open system, we sought to monitor the state of FUSE in active linear templates. Therefore FUSE was embedded in a 1-kilobase (kb) segment between divergently

**Figure 2** Two-dimensional agarose gel analysis of supercoil-dependent DNA structural transitions in +FUSE and –FUSE midi-circles. (**a**,**b**) Electrophoresis of DNA midi-circles (1 kb) was carried out in the first dimension from top to bottom without (**a**) or with 1.5 mM $Mg^{2+}$ (**b**), and in the second dimension from left to right in the presence of ethidium bromide. Left, autoradiographs of a Southern blot of two-dimensional gels. Right, a diagrammatic overlap of the +FUSE and –FUSE topoisomer curves, including the writhing numbers ($\omega$) of the individual topoisomer spots; cyan spots, topoisomers of the +FUSE circles that migrate differently than their –FUSE counterparts. The $Mg^{2+}$-dependent transition from B-DNA to an unusual structure is detectable as a ripple (arrows) in the otherwise monotonic distribution of topoisomers. The spot labeled "n" corresponds to nicked DNA. Bottom, schematic explanation of the discontinuity in topoisomer migration resulting from a supercoil-dependent DNA structural transition.



transcribed T3 and T7 promoters in a 3-kb fragment and a pulse of potassium permanganate was added both to probe DNA structure and to stop transcription. Standard footprint analysis of the modified bases was complicated because the 'single-hit' conditions normally used to probe DNA structure inhibited transcription by oxidizing the template DNA and RNA polymerase protein. Stopping transcription relaxes DNA and reverts FUSE to B-DNA. Therefore transcription reactions were terminated under 'multiple-hit' conditions using a 'shot' of potassium permanganate to modify melted DNA. This modification destabilizes the helix at the modified bases and so marks single-stranded character even after removal of the destabilizing torque. The FUSE region contains an AvaI site on the cusp of the first and second melted regions, outside of the segment that forms H-like-DNA (**Fig. 3a**). To assess the degree of melting at the FUSE, the modified DNA recovered from the transcription reactions was cleaved with AvaI; DNA melting and the consequent covalent modification of this site was expected to leave AvaI resistance as a record of prior FUSE single-strandedness. Using this scheme, ongoing transcription rendered a substantial portion of the linear template (~10%) DNAs resistant to AvaI (**Fig. 3b**, right panel).

To calibrate this assay, circular DNAs with defined $\langle\sigma\rangle$ were permanganate-modified in presence or absence of $Mg^{2+}$, linearized

and tested for AvaI resistance inside the FUSE. Without $Mg^{2+}$, a sharp transition to resistance occurred as $\langle\sigma\rangle$ was increased from –0.08 to –0.09 (**Fig. 3b**, left panel). With $Mg^{2+}$, the FUSE remained fully AvaI sensitive irrespective of permanganate treatment (data not shown). Full cleavage of FUSE in stably supercoiled DNA, modified in the presence of $Mg^{2+}$, indicated that the alternative structure detected by chemical and topological analysis (**Figs. 1** and **2**) did not include that AvaI site. Molecules made resistant to AvaI by ongoing transcription were not likely to have adopted the AvaI-sensitive alternative H-like conformation present in stably supercoiled DNA. Therefore, despite the presence of $Mg^{2+}$ in the transcription reaction, dynamic supercoiling supported alternative structures less efficiently than did time-independent torsional stress. To form alternative structures, DNA melting of a sufficient number of base pairs must be followed



**Figure 3** FUSE melting as a torsional strain gauge. (**a**) Top, map of the linear FUSE-containing DNA template used to trap dynamically stressed FUSE in an unwound state. The distance between T3 and T7 promoters is 1.1 kb. The length of the transcribed regions for each RNA polymerases is 1.3 kb. Bottom, structural elements in FUSE. Green bar, region corresponding to H-like-DNA according to the data presented here. The regions predicted to be easily melted by supercoiling[14] and verified with the data presented here are bracketed. (**b**) Restriction enzyme mapping of the strain-dependent chemical modifications inside the FUSE sequence. Topoisomers of circular or linear pFLIP-FUSE DNA (left), or linear DNA under conditions with (+) and without (–) transcription (right), were briefly treated with potassium permanganate. Subsequent linearization of the circular DNA was used to relieve all superhelical stress. DNAs were then cleaved with AvaI. Resistance to cleavage by AvaI revealed the single-stranded character within FUSE captured by chemical modification.
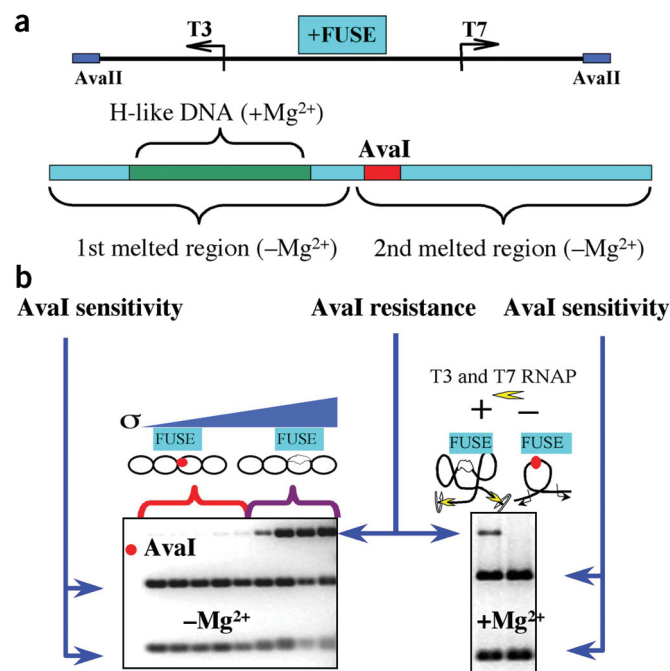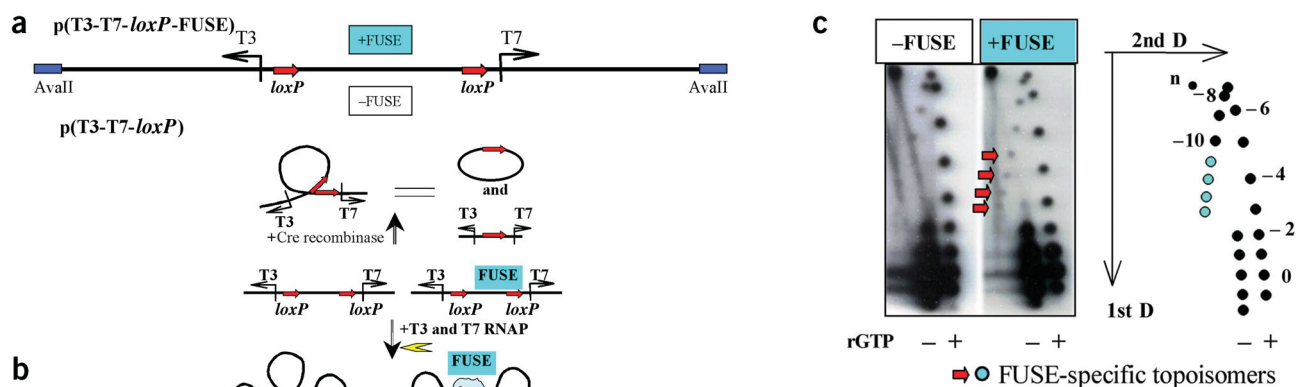
**Figure 4** An experimental approach to capture and measure dynamic DNA supercoiling during transcription. (**a**) Substrates to probe dynamic supercoiling during transcription on linear DNAs. Plasmids p(T3-T7-*loxP*) and p(T3-T7-*loxP*-FUSE) contain two directly repeated target sites (*loxP*) for the Cre recombinase located upstream of divergently transcribed T3 and T7 promoters. The length of otherwise identical –FUSE or +FUSE DNA sequences nested between the *loxP* sites is 1.0 kb. AvaII was used to linearize plasmids; the lengths of the transcribed regions are 1.3 kb. (**b**) A scheme to detect dynamic DNA supercoiling during transcription. Site-specific recombination of linear DNA produces the thermal distribution of relaxed circles and a shortened linear fragment. Transient torsional stress introduced into the DNA as result of transcription is preserved by recombination as permanent topological change within the excised circles resulting in supercoils. Thus, a topological analysis of recombination products yields information about the structure of the substrate DNA at the moment of recombination. If the FUSE is conformationally changed due to dynamic supercoiling then it will act as a capacitor absorbing supercoils, and circles with FUSE should contain more supercoils then circles without FUSE. (**c**) Two-dimensional gel topological analysis of recombination products on linear DNA without (–) and with (+) transcription. Lane –, without transcription and with 2% (w/v) PEG 8000. Lane + transcription started with rGTP (1 mM). Electrophoresis was carried out in the first dimension from top to bottom in the presence of 7.8 µg ml$^{-1}$ of chloroquine, and in the second dimension from left to right in the presence of 0.5 µg ml$^{-1}$ of ethidium bromide. Under these conditions, a topoisomer with $\omega = -8$ migrates as if it was fully relaxed. At right, individual topoisomers are identified as labeled spots. The position of the spots corresponding to relaxed circle, $\omega = 0$, were located according to one-dimensional gel topological analysis (data not shown). Inclusion of the FUSE yields additional spots (cyan). Quantitative analysis showed that equal amounts of –FUSE and +FUSE midi-circles were recovered as result of Cre recombination under conditions with and without transcription.

by an intramolecular reorganization. This second step is slow relative to the diffusion of torsional stress; the characteristic time for non-B-DNA formation in supercoiled DNA is ≥1 min[23,26,27]. Unless torsional stress is sufficiently prolonged, the dynamic melting of FUSE in the presence of magnesium during transcription would be unlikely to reconfigure into another structure. At the same time, the dynamic supercoiling forces may support a range of FUSE conformations from a melted bubble to other non-B-DNA structures.

### Supercoiling between promoters in linear DNA

The experiments above used ongoing transcription driving focal changes in DNA conformation to infer changes in global DNA topology. Although the interrelationship between DNA conformation and topology is precisely defined by a static linking number within fixed topological boundaries, the dynamic case with open boundaries is less well studied. An open system may better approximate the situation *in vivo*, where dynamic forces are distributed within large domains, modified by fluctuating topological barriers, and opposed by topoisomerases. We devised an assay to trap and measure directly the transient stresses propagated through regions upstream of active promoters (the scenario depicted in **Fig. 4b**). *loxP* sites were placed between (and so upstream of) divergently transcribed T3 and T7 promoters in a linear DNA fragment. Otherwise, identical +FUSE or –FUSE segments were nested between the *loxP* sites (**Fig. 4a**). Cre recombinase (i) operates efficiently on both relaxed and supercoiled DNA, and (ii) conserves linking number during site-specific recombination ($\Sigma$ Lk products = $\Sigma$ Lk substrate)[28,29], therefore excision and circularization by Cre recombinase of the 1.0-kb DNA segment

interposed between the *loxP* sites creates a new topological domain, trapping the torsional stress residing in the DNA segment at the instant of ring closure. (An earlier variation on this assay demonstrated that transcriptionally generated torsion licenses recombination by the supercoil-dependent γδ-resolvase. Owing to the particular properties of γδ-resolvase and the design of the templates, the magnitude and the distribution of the transient stress could not be measured or assessed[11,12].) Recombination of untranscribed linear DNA would be expected to yield both a population of midi-circle topoisomers thermally distributed around the fully relaxed state, and a shortened linear segment. During transcription from divergent promoters, Cre recombination should recover a population of midi-circles shifted from the thermal distribution according to the amount of torsional stress transiting through the DNA at the moment of ring closure. If FUSE is driven into a non-B-DNA conformation by torsional stress, then it will act as a capacitor absorbing supercoiling, and +FUSE circles should contain more supercoils than –FUSE (**Fig. 4b**).

The $\langle\sigma\rangle$ of excised circles (**Fig. 4c**) was assessed by two-dimensional gel electrophoresis using chloroquine in the first dimension. Recombination of untranscribed linear DNA yielded the expected midi-circles, which were thermally distributed around the most relaxed topoisomer. When transcription was activated by adding rGTP, a considerable portion of the midi-circles was converted to more highly supercoiled forms. Superhelical densities for the circles excised during transcription ranged beyond –0.1, exceeding the value driving FUSE distortion. Including FUSE increased the number supercoils (14 versus 10) trapped by recombination; this observation indicates that at least 40 bp of FUSE, presumably the entire first melted

**Figure 5** Transcription from a single promoter supports supercoiling of upstream DNA. Two-dimensional gel analysis of the topology of the recombination products derived from the linear DNA template during transcription from a single promoter. Lane –, transcription was prevented by the omission of rGTP in the reaction. Lane +, transcription from T3 promoter was activated by adding rGTP (1 mM) to the reaction mixture. Site-specific recombination and topological analysis of the reaction products were carried out exactly as described in the legend to **Figure 4**, but the reactions contained only one RNA polymerase (T3 RNAP). Substrates for the reactions were AvaII-linearized p(T3-T7-*loxP*) (left) or p(T3-T7-*loxP*-FUSE) (right). The drawing on the right assigns ω to individual spots. Inclusion of the FUSE yields additional spots (cyan). Quantitative analysis showed the equal amounts of –FUSE and +FUSE midi-circles were recovered as result of Cre recombination under conditions with (+) and without (–) transcription.



: FUSE-specific topoisomers
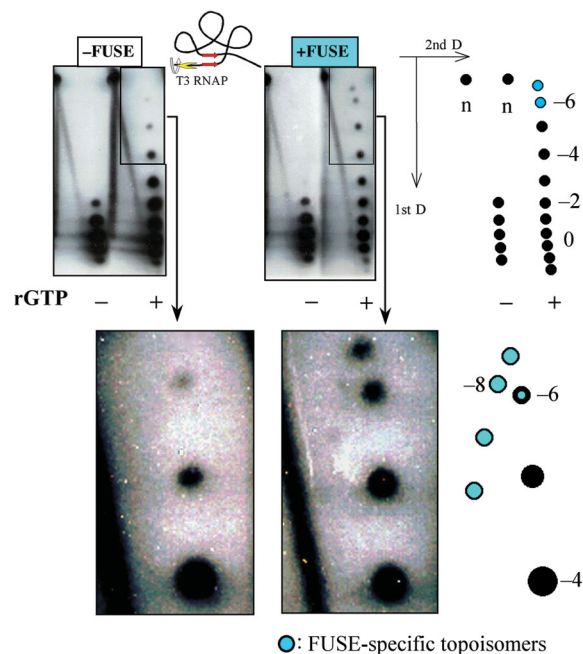
region (**Fig. 2a**), were unwound at the instant of recombination. Therefore, despite the presence of $Mg^{2+}$, obligatory for transcription, dynamic supercoiling did not efficiently support the isomerization of FUSE into H-like DNA.

## Dynamic supercoils upstream of a single active promoter

The experiments above used templates bearing two active, divergent promoters. (This situation is not very artificial, because the divergent configuration of promoters is a very common feature of mammalian genomes associated with 10–30% of genes, and possibly with CpG islands[30,31].) To determine whether transient stress is unique to this arrangement, the level of dynamic supercoiling was measured on a linear template with only one active promoter. This case may mimic the generation of dynamic supercoiling by DNA-tracking RNA polymerases *in vivo* at widely spaced promoters where torsional stress is dissipated by diffusion or topoisomerases. Addition of only one RNA polymerase (T3 RNAP) to the transcription-Cre recombinase reaction still yielded highly supercoiled midi-circles (**Fig. 5**). This population was more prominent with +FUSE versus –FUSE circles. Inclusion of the FUSE also increased the range of supercoils (10 versus 6, compared with 14 versus 10 for divergent transcription) dynamically trapped by recombination. Because the torsional energy stored in a spring is $1/2\,k\theta^2$, θ being the twist angle, these data suggest that each promoter in case of divergent transcription contributes half the elastic energy stored in the DNA fiber. These results confirm that melted FUSE serves as a capacitor storing torsional energy. Because transcription from a single promoter supported a much smaller fraction of molecules exceeding the $\langle\sigma\rangle = -0.07$ threshold for FUSE melting, very few molecules should become AvaI-resistant after permanganate treatment; this prediction was confirmed (data not shown).

## Parameters controlling dynamic supercoils

The intensity of negative supercoiling in the midi-circles excised during transcription was notable. Additional experiments confirmed that these effects resulted from ongoing transcription and not looping, folding or twisting of the DNA segment between the T7 and T3 promoters.

First, if this is generated by a dynamic process, then the number of supercoils in recovered midi-circle depends on the transcription rate, and faster transcription should intensify supercoiling. Without rGTP, no RNA synthesis occurred, and only thermal supercoils were recovered. Increasing rGTP concentration increased RNA production (**Fig. 6a**, left) and progressively pumped more supercoils into the midi-circle (**Fig. 6a**, right). If the dynamic torsional stress transiting through the template rises with the rate of transcription, then the probability of FUSE melting should also depend on the intensity of transcription.

To ascertain whether FUSE melting is tuned to the rate of transcrip-

tion, active templates in transcription reactions using graded concentrations of rGTP were modified with permanganate and tested for AvaI cleavage, as in **Figure 3**. This assay (**Fig. 6b**) revealed that the fraction of AvaI-resistant DNA paralleled the increase in highly supercoiled circles (compare bar graphs in **Fig. 6c**). Direct visualization of the modified bases by footprinting (**Supplementary Fig. 1** online), though complicated by multiple-hit conditions, nevertheless revealed an rGTP-dependent increase in FUSE reactivity. Thus both direct and indirect assays for torsional stress and strain reveal that active linear templates are functionally highly supercoiled.

Second, whereas limiting rGTP during transcription limits the generation of torsional stress, maneuvers that retard the dissipation of this stress from the free ends of the template should increase dynamic supercoiling during transcription. As the viscosity of the reaction media was increased with the addition of PEG 8000 (maximally to 5% (w/v)), the transcriptionally engaged polymerases cranked the template against greater frictional resistance, and so supercoiling intensified (**Supplementary Fig. 2** online). Whereas inactive templates treated with permanganate in the presence of PEG were fully cleaved by AvaI, under these same conditions, a considerable fraction of the transcriptionally active FUSE-containing templates acquired AvaI resistance. As expected, increasing PEG concentration increased the proportion of AvaI-resistant molecules (**Supplementary Fig. 2** online).

Third, if the hydrodynamic drag of the newly synthesizing RNA retards counter-rotation of the RNA polymerase and template, then the yield of supercoils in the excised circles should depend on nascent transcript length. Reducing the length of the transcribed regions decreased the friction on active transcription complexes and diminished the degree of supercoiling in the excised circles (**Supplementary Fig. 2** online). Fourth, including RNase in these reactions to shave the nascent RNAs off the elongating transcription complexes abrogated the recovery of supercoiled midi-circles (**Supplementary Fig. 2** online) and the production of AvaI-resistant +FUSE DNA (data not shown), as predicted previously[1] (and as seen using γδ-resolvase and *res* sites placed downstream of the transcription start site in topologically bounded templates[11]).
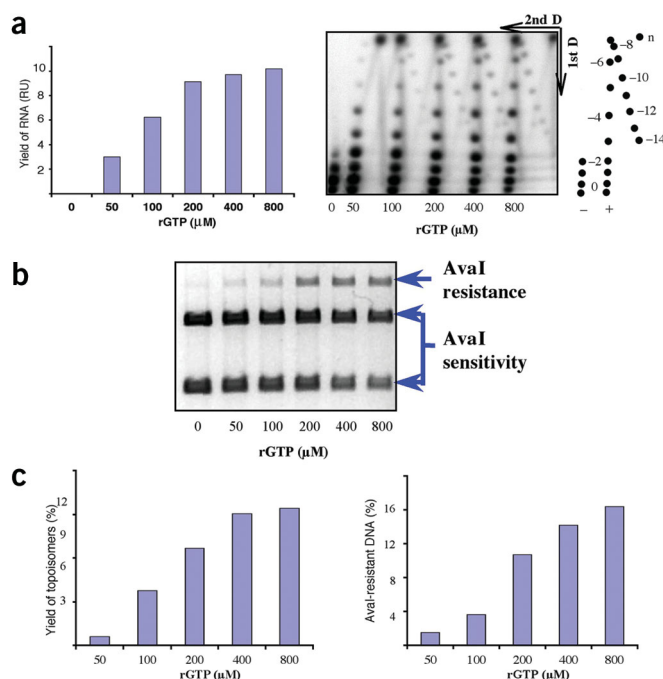
**Figure 6** Influence of the transcription rate on the intensity of supercoiling and FUSE melting. (**a**) Transcription was activated upon addition of the indicated concentrations of rGTP to the reactions. Site-specific recombination and topological analysis of the reaction products were carried out exactly as described in the legend to **Figure 4**. The substrate for the recombination reaction was p(T3-T7-*loxP*-FUSE) linearized by AvaII. Left, aliquots of the reaction were run on an agarose gel, and RNA yield in relative units (RU) as a function of rGTP concentration was quantified using ImageQuant. Middle, autoradiographs of a Southern blot for two-dimensional gels. Right, diagram indicating the writhing number ($\omega$) of individual topoisomer spots. (**b**) AvaI-resistance analysis of the FUSE conformation was done as described in the legend to **Figure 3**. (**c**) Left, quantitative analysis of the recovery of circles more highly supercoiled than $\sigma = -0.07$ as a function of transcription rate assessed with a phosphorimager. After background correction, the sum of the signals from each topoisomer band ($\omega \leq -7$) was plotted against the rGTP concentration. The yield of recombination products was normalized for each lane. Right, extent of DNA modification at AvaI site as uncut / (cut + uncut) versus rGTP concentration.

part of the FUSE becomes melted. At samples 13 and 14 melting is complete and the entire FUSE is single-stranded. These topologically defined circles served as probes for FBP in electrophoretic mobility shift assays (**Fig. 7**). The relationship between $\langle\sigma\rangle$ and alternative FUSE conformations enabled correlation between FBP binding and DNA structure. The FBP-FUSE interaction first yielded a diffuse band with a downward smear indicative of in-gel dissociation of a low-mobility, low-stability complex (**Fig. 7b**; lanes 8–10). At higher $\langle\sigma\rangle$, the stabilized FBP–FUSE complex migrated as a discrete band (**Fig. 7b**; lanes 11–14). Binding was not detected in circles lacking FUSE. In the presence of $Mg^{2+}$, which stabilizes both B- and the H-like DNAs, FBP did not bind to FUSE (**Figs. 1** and **2**), although the FBP interaction with FUSE oligonucleotides was not affected (data not shown). These results suggest that binding of FBP to melted FUSE competes with an alternative $Mg^{2+}$-dependent non-B-DNA conformation that is sustained by torsional stress.

In the presence of physiological $Mg^{2+}$, transient as opposed to stable supercoiling forces may more effectively expose the determinants in single-stranded FUSE necessary to bind FBP.

Finally, recovery of supercoils in Cre-excised circles rapidly ceased in <10 s after halting transcription by depleting ATP with apyrase, indicating the evanescent nature of this dynamic stress (**Supplementary Fig. 3** online).

These results show that the dynamic supercoils captured by Cre and FUSE's single-stranded character in linear templates result from transcription-generated dynamic stress. Furthermore, they reveal a marked difference in the FUSE response to dynamic torsional stress versus stable supercoiling. And finally, they discount the possibility that the high level of supercoiling measured during coupled transcription and recombination is due to hindrance of twist diffusion by a Cre recombinase–DNA complex.

## DNA conformation licenses FUSE-FBP interaction

The topoisomer and conformation preferences of FBP for binding to FUSE were examined as an example of supercoil-induced recruitment of regulatory proteins to duplex destabilized sites. Pools of +FUSE and –FUSE minicircles with defined $\langle\sigma\rangle$ were constructed by self-ligation of $^{32}P$ end-labeled 630-bp fragments followed by topoisomerase treatment with increasing ethidium bromide levels. This experiment provides information about FUSE structure for each sample. In samples 1–6, the conformation of the FUSE is B-form; between samples 7 and 10 the first part of FUSE is melted, from samples 11 to 13, the second

## DISCUSSION
## Conformational changes

Dynamic supercoiling driving conformational changes in specific sequences has many ramifications. First, such changes may report the status of ongoing gene activity. FUSE melts in response to the transient generation of torque by ongoing transcription. Because dynamic
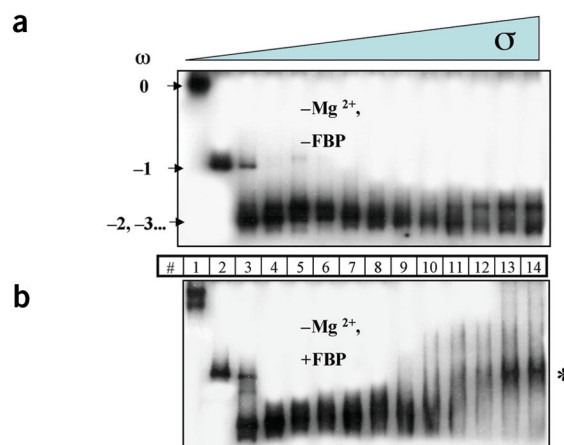
**Figure 7** Binding of FBP to minicircle topoisomers. Electrophoresis of +FUSE minicircles (630 bp) with increasing (from 1 to 14) supercoiling density. (**a**,**b**) DNAs were preincubated for 30 min in DNA-binding buffer without (**a**) or with (**b**) recombinant FBP. DNA-binding buffer and electrophoresis buffer did not contain $Mg^{2+}$. The number of superhelical turns in the DNA of each sample in the absence of structural transitions was assigned on the basis of the DNA electrophoretic mobility in gels containing different concentrations of chloroquine or ethidium bromide (data not shown). The addition of FBP causes slower minicircle migration due to formation of protein–DNA complex starting with sample 8, and finally resulting in the appearance of a new discrete band (asterisk). No binding of FBP to topoisomers of control –FUSE molecules was observed (data not shown).

supercoiling is sensitive to the rate of transcription, and melting at FUSE is associated with a sharp threshold of torsional stress, FUSE has the potential to serve as a real-time sensor of ongoing transcription. Once melted, the FUSE is available for recognition by FBP and its opposing repressor FIR[17,32]; through their transcriptional effector domains, these proteins may close a feedback loop responding in real time to local ongoing transcription (**Fig. 8**). This sort of regulation is likely to be relevant for the regulation of short-half-life, low-abundance mRNAs. The delays imposed by RNA processing, mRNA transport, translation, nuclear import, assembly into complexes, protein modifications and targeting back to the active promoter, coupled with the noise intrinsic to low level transcription, limit the kinetics and precision attainable by end-product feedback of proteins on RNA synthesis. Second, another consequence of supercoil-provoked DNA melting is the creation of flexural and torsional hinges in chromatin. Owing to the much higher flexibility of single-stranded DNA to bending and twisting, these hinges could grant access of factors bound at upstream locations to sites from which they are otherwise stereochemically restricted[6]. Third, transcription-induced melting allows a single segment of DNA to bind one set of factors when it is a duplex and another when it is single-stranded. Fourth, because nucleosomes are wrapped with slightly overwound B-DNA, and do not bind single strands, transcriptionally provoked melting at a sensitive sequence is likely to exclude nucleosomes and to influence the architecture of the embracing chromatin[33]. Owing to the vectorial nature of dynamic supercoils, melting at sensitive sites is expected to be position-dependent. Increasing the distance from the torque generator, or interposing other stress-absorbing elements, would be predicted to alter the kinetics and probability of induced conformational changes[34]. Sandwiching a stress-sensitive site between two equally active divergent promoters would tend to buffer this position-dependence. The kinetics of stress transmission is also likely to influence the distribution between the alternative conformations available to a susceptible segment. Sequences that can assume noncanonical structures are often present in the regulatory regions of genes. These structures have generally been investigated only at equilibrium in stably supercoiled DNA. Our results suggest that the lifetimes of dynamic supercoil-melted regions may be too short to adopt stable alternative structures.

## Chromatin structure

*In vivo*, dynamic supercoils must contend with chromatin; how do nucleosomes influence the transmission of torsional stress? Packaging into nucleosomes shortens and thickens DNA fibers. Because rotary friction is linearly dependent on the length, but has a higher-order dependence on radius, chromatin is harder to spin after nucleosomal compaction. This increased friction would be expected to delay the dissipation of torsional stress, and to exaggerate dynamic supercoiling[35]. Other maneuvers that deform the helical axis, such as DNA-bending, would also be expected to focus dynamic torsional stress between the source (the transcribing polymerase) and the deformation[9]. If, as structural studies indicate, the encircling DNA is secured to the nucleosomal surface, then the dynamic torsional stress of transcription would be dispersed in a saltatory fashion between successive linker segments, bypassing the nucleosomal DNA.

The transient superhelical stress of transcription in these experiments approximates the levels involved in chromatin remodeling[36,37]. Hence the chromatin remodeling machinery may be modulated by the physical stress of nearby transcription. Because of the directional nature of torque propogation, whether dynamic supercoils would oppose or enhance chromatin remodeling would depend on the architectural details of the involved chromatin. Similarly, loosening the grip of his-
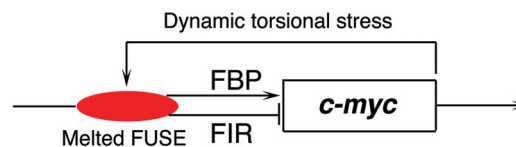


**Figure 8** FUSE, FBP and real-time regulation of transcription. FBP bears a potent activation domain that is involved in the regulation of *c-myc* transcription through binding to the single-stranded FUSE. FUSE and FBP also bind the repressor FIR[17,32]. From the present results, FUSE is a supercoil-sensitive element that changes conformation in response to the dynamic torsional stress resulting from transcription. Thus FUSE melting has the capacity to serve as a real-time reporter of promoter activity and to actuate FBP and FIR effector function to regulate ongoing transcription.

tone tails by acetylation would change the kinetics of the absorption and transmission of dynamic supercoils. The efficiency for transmitting this stress through chromatin will inevitably be highly context-dependent. The imposition of permanent or transient (such as frictional) barriers, and the opening and closing of loops between DNA-bound factors, may govern the kinetics for dissipating dynamic supercoils. How the architectural features of chromatin influence the dynamics of torsional stress transmission remain largely unexplored.

## Promoter activity and arrangement

Dynamic under- or overtwisting of DNA may assist or oppose transcriptional elongation by reducing or exaggerating the number and/or duration of pauses and alter the probability of backtracking or extension[38]. Divergent, closely spaced promoters are common in mammalian genomes and often separated by as few as 100 bp[30,31]. Lacking special mechanisms to isolate or remove transcriptionally generated stress, the torque diffusing away from one promoter would pass through its neighbor. This torque would facilitate melting at the next upstream start site, and so the activity of closely juxtaposed promoters may be mechanically coupled[39]. The discovery of numerous noncoding RNA genes near the promoters of protein-coding genes may presage frequent use of dynamic supercoiling as a regulatory device[40].

Because RNA polymerase is the most forceful cellular motor, transcription is inevitably coupled with the dynamic pumping of torque into the double helix. The consequences of operating such a powerful molecular machine have implications for a variety of DNA transactions[41].

## METHODS

**Enzymes and reagents.** Enzymes and chemicals were obtained from New England Biolabs, Stratagene, Amersham Pharmacia Biotech, Roche Molecular Biochemicals and Sigma.

**DNA.** Plasmid p(T3-T7) was constructed from pUC-19 by inserting promoter sequences for T7- and T3-RNAP into the HindIII and EcoRI sites, respectively. p(T3-T7-*loxP*) included a blunt-ended 1.0-kb NotI fragment from pDNR-1r (Clontech) containing *loxP* sites in direct orientation cloned in the SmaI site of p(T3-T7). FUSE-91 sequence, HindIII-SpeI fragment from the pUC-FUSE/T7 (made by H.-J.C.), was blunt end–cloned into the SpeI-XmaI sites of p(T3-T7-*loxP*). Templates for transcription and substrates for Cre recombinase were prepared by linearization of plasmids with AvaII, or with XmnI and AflIII restriction enzymes. *In vitro* generation of midi-circles was achieved by incubation of linear DNAs with Cre recombinase recommended by the manufacturer. pFLIP is a 1350-bp BspHI-NdeI fragment of pUC-19 inserted into a 1000-bp AvrII fragment of pEGFP-C1 (Clontech). This procedure swapped *amp-r to kan-r*, removing a supercoil-dependent DNA-destabilization region associated with the former gene[42,43]. pFLIP-FUSE includes a 113-bp HindIII-SpeI fragment from the pUC-FUSE/T7 blunt end cloned between the SacI-EcoRV sites of pFLIP. +FUSE plasmid was constructed by blunt-end cloning FUSE-

containing HindIII-SpeI fragment from the pUC-FUSE/T7 into the KpnI-BsmBI sites of pDNR-1r. –FUSE plasmid is pDNR-1r with no insert between sites AvrII and BsmBI. Plasmid DNAs were isolated by alkaline lysis followed by equilibrium centrifugation in cesium chloride–ethidium bromide gradients.

**Generation of topoisomers.** Topoisomers with defined superhelical densities were prepared by the topoisomerase-ethidium bromide technique[44]. The average number of supercoils and superhelical density of each population of topoisomers was determined using agarose or polyacrylamide electrophoresis in the presence of chloroquine[45,46].

**Gel electrophoresis topological analysis.** For the first dimension, electrophoresis of topoisomer mixtures or reaction samples was carried out on 3% (w/v) agarose in TAE buffer, pH 7.6, with or without 1.5 mM MgCl$_2$. Buffer contained or did not contain 7.8 μg ml$^{-1}$ of chloroquine. TAE buffer, pH 8.3, for electrophoresis in the second dimension contained 0.5 μg ml$^{-1}$ of ethidium bromide. The distribution of topoisomers was visualized by Southern blotting and autoradiography. Quantification of the bands was done with ImageQuant (Molecular Dynamics). The superhelical density required for, and absorbed by, unusual DNA structure formation was determined by counting the number of superhelical turns ($\omega$) at which structural transition occurred. The superhelical tension $\sigma$ required for structural transition of the DNA was calculated using the formula: $\sigma = -10.5\omega / N$, where $N$ = the number of base pairs in the DNA.

**Mapping of DNA unpairing at FUSE.** Each sample, containing 5 μg of pFLIP-FUSE DNA at particular average supercoiling density in TAE buffer, pH 7.6, with (1.5 mM MgCl$_2$) or without Mg$^{2+}$ was treated with KMnO$_4$ (6 mM) at 37 °C for 3 min. The reaction was quenched by the addition of 2-mercaptoethanol to 1.0 M. The DNA was digested with NotI and SpeI, and the FUSE-containing fragment was purified from the agarose gel. In the case of DNAs chemically modified during transcription, the FUSE-containing fragment was recovered after digesting the template with NdeI and NcoI. To end-label the bottom strand of FUSE, the DNA was 5′-end-labeled with [γ-$^{32}$P]ATP using T4 polynucleotide kinase, and then digested with HindIII for analysis of supercoiled plasmids or with EcoRV to analyze transcription templates. The DNA was treated with piperidine (1 M) at 90 °C for 30 min to cleave permanganate adducts, and the samples were analyzed by 7% and 12% (w/v) sequencing gels.

**Restriction enzyme sensitivity assay.** Each sample, containing either 0.6 μg of pFLIP-FUSE in 50 mM Tris-HCl, pH 7.6, 33 mM NaCl, 1 mM DTT, 200 μg ml$^{-1}$ BSA, with or without 10 mM MgCl$_2$ at a particular supercoiling density, or transcription reaction mixture with 0.6 μg of linearized p(T3-T7-*loxP*-FUSE), was treated with KMnO$_4$ (25 mM) at 37 °C for 10 s. The reaction was quenched by the addition of 2-mercaptoethanol to 1.0 M. Circular DNAs were then linearized by NcoI restriction enzyme. After this step, linear molecules were cleaved with AvaI. Fragments were electrophoresed in 1% w/v agarose gels, stained with ethidium bromide, photographed with a Kodak EDAS120 camera, and quantified with ImageQuant.

**Gel retardation analysis.** The 625-bp NotI fragment of +FUSE and the 629-bp NotI fragment of –FUSE were dephosphorylated and end-labeled with [γ-$^{32}$P]ATP using T4 polynucleotide kinase. The fragments were separately ligated by T4 DNA ligase. Circular DNA was identified on the basis of its fast electrophoretic mobility on an agarose gel containing ethidium bromide, and extracted from the gel by electroelution. Topoisomers of –FUSE and +FUSE minicircles were generated as described above. For gel retardation analysis, topoisomer samples and recombinant FBP purified from insect cells[32] were mixed (at a molar protein/DNA ratio of 3:1) in the DNA-binding buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 0.2 mM EDTA, 1 mM DTT, and 10 % (v/v) glycerol, in the presence of 50 μg ml$^{-1}$ poly(dI-dC) and 1 mg ml$^{-1}$ BSA) with (1.5 mM MgCl$_2$) or without magnesium. The samples were incubated for 30 min at room temperature, electrophoresed on a 4% (w/v) polyacrylamide gel, and visualized by autoradiography.

**In vitro Cre recombination and transcription.** Recombination and transcription were assayed at 37 °C in 50 mM Tris-HCl, pH 7.6, 33 mM NaCl, 10 mM MgCl$_2$; 1 mM DTT; 200 μg ml$^{-1}$ BSA; 1 mM ATP, CTP and UTP. Reaction mixtures (50 μl) contained 0.6 μg of DNA, 40 U T7 and/or 40 U

T3 RNAP, and PEG 8000 varied from 0% to 5% (w/v). Transcription was started by adding rGTP (0–1 mM). Cre recombinase (3 U) was added last, at 3 min after preincubation of the mixture at 37 °C, and the reaction was allowed to proceed for another 10 min. In the case of the restriction enzyme sensitivity assay, Cre recombinase was omitted. To monitor transcription rates, aliquots of the reaction were run on a 1.0% (w/v) agarose gel. Products of recombination were analyzed by one-dimensional and/or two-dimensional gel electrophoresis as described above.

*Note: Supplementary information is available on the Nature Structural & Molecular Biology website.*

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